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Dorfin prevents cell death by reducing mitochondrial localizing mutant superoxide dismutase 1 in a neuronal cell model of familial amyotrophic lateral sclerosis

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Abstract

Dorfin is a RING-finger type ubiquitin ligase for mutant superoxide dismutase 1 (SOD1) that enhances its degradation. Mutant SOD1s cause familial amyotrophic lateral sclerosis (FALS) through the gain of unelucidated toxic properties. We previously showed that the accumulation of mutant SOD1 in the mitochondria triggered the release of cytochrome *c*, followed by the activation of the caspase cascade and induction of neuronal cell death. In the present study, therefore, we investigated whether Dorfin can modulate the level of mutant SOD1 in the mitochondria and subsequent caspase activation. We showed that Dorfin significantly reduced the amount of mutant SOD1 in the mitochondria, the release of cytochrome *c* and the activation of the following caspase cascade, thereby preventing eventual neuronal cell death in a neuronal cell model of FALS. These results suggest that reducing the accumulation of mutant SOD1 in the mitochondria may be a new therapeutic strategy for mutant SOD1-associated FALS, and that Dorfin may play a significant role in this.

Keywords: amyotrophic lateral sclerosis, Dorfin, mitochondria, neuronal cell death, superoxide dismutase 1, ubiquitin ligase.

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by selective death of motor neurons. Approximately 10% of ALS cases are familial (FALS). Missense mutations in the gene coding superoxide dismutase 1 (SOD1) are responsible for approximately 20% of FALS cases (Rosen *et al.* 1993; Hirano 1996) through the gain of unelucidated toxic properties (Yim *et al.* 1996).

Many reports have documented that the mitochondria are involved in the pathogenic process in mutant SOD1-associated FALS. Mitochondrial degeneration, including swelling, dilatation and vacuolization, is an early characteristic pathological feature of FALS and FALS transgenic (Tg) mice models with SOD1 mutations (Dal Canto and Gurney 1994; Wong *et al.* 1995; Hirano 1996; Kong and Xu 1998; Jaarsma *et al.* 2000; Higgins *et al.* 2003). Recently, it was demonstrated that SOD1, considered to be a cytosolic enzyme, exists in the mitochondria (Sturtz *et al.* 2001; Okado-Matsumoto and Fridovich 2001; Higgins *et al.* 2002), and that the mitochondrial vacuoles in mutant SOD1 Tg mice were lined with mutant SOD1 (Jaarsma *et al.* 2001; Higgins *et al.* 2003). Many studies have suggested that the programmed cell death (PCD) pathway contributes to motor neuron death in FALS (Durham *et al.* 1997; Martin 1999; Li *et al.* 2000; Pasinelli *et al.* 2000; Guégan *et al.* 2001; Kriz *et al.* 2002; Raoul *et al.* 2002; Zhu *et al.* 2002). Moreover, we previously reported that accumulation of mutant SOD1 in the mitochondria triggered the release of mitochondrial cytochrome *c*, which subsequently activated the caspase cascade and induced neuronal cell death (Takeuchi *et al.* 2002a). Taken together, these results suggest that the accumulation of mutant SOD1 in the mitochondria is critical in the pathogenesis of mutant SOD1-associated FALS.

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Abbreviations used: ALS, amyotrophic lateral sclerosis; COX, cytochrome *c* oxidase; DMEM, Dulbecco's modified Eagle's medium; E3, ubiquitin ligase; EGFP, enhanced green fluorescent protein; FALS, familial amyotrophic lateral sclerosis; MTS, 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PCD, programmed cell death; PI, propidium iodide; SOD1, superoxide dismutase 1; Tg, transgenic.

Dorfin is the product of a gene that we cloned from the anterior horn tissue of the human spinal cord (Niwa *et al.* 2001); it contains a RING-finger/IBR motif (Niwa *et al.* 2001) at its N-terminus. It was reported that a distinct subclass of RING-finger/in-between RING-fingers (IBR) motif-containing proteins represents a new ubiquitin ligase (E3) family that interacts specifically with distinct ubiquitin-conjugating enzymes (Moynihan *et al.* 1999; Ardley *et al.* 2001). Dorfin is a juxtanuclearly located E3 that ubiquitylates various SOD1 mutants derived from patients with FALS, and enhances the degradation of mutant SOD1 (Niwa *et al.* 2002). Whether Dorfin can modulate the protein level of mutant SOD1 in the mitochondria, and the subsequent activation of the mitochondrial caspase cascade, is an important and interesting question.

Here we show that Dorfin significantly reduced the amount of mutant SOD1 in mitochondria, the release of cytochrome *c* from mitochondria into the cytosol and the subsequent activation of the caspase cascade, thereby preventing the eventual neuronal cell death in a neuronal cell model of FALS. These results suggest that reducing mutant SOD1 in the mitochondria may be a useful strategy for the treatment of mutant SOD1-associated FALS, and that Dorfin might play a significant role in this.

Materials and methods

Plasmids

Non-organelle-oriented plasmids expressing the enhanced green fluorescent protein (EGFP)-tagged human SOD1 (wild type, mutant G93A, and G85R) were described previously (Takeuchi *et al.* 2002a,b). These vectors express SOD1-EGFP fusion proteins ubiquitously in each organelle (Takeuchi *et al.* 2002a). They were designated Cyto-WT, Cyto-G93A and Cyto-G85R respectively. Mitochondria-oriented plasmids expressing EGFP-tagged human SOD1 (wil dtype, mutant G93A and G85R) with mitochondrial localizing signals were generated as described previously (Takeuchi *et al.* 2002a). These vectors express SOD1-EGFP fusion proteins mainly in the mitochondria (Takeuchi *et al.* 2002a). They were designated Mito-WT, Mito-G93A and Mito-G85R respectively. The plasmid pcDNA3.1/HisMax-Dorfin, which expresses Xpress-tagged Dorfin, was also described previously (Niwa *et al.* 2001). As a control, we used pCMV-β vector expressing LacZ (Clontech, Palo Alto, CA, USA). All constructs used here were confirmed by DNA sequence analysis.

Cell culture

Mouse neuroblastoma cell line Neuro2a cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen Corp.) as described previously (Takeuchi *et al.* 2002b). They were cultured on Laboratory-Tec II four-well chamber slides (Nalge Nunc International, Rochester, NY, USA) coated with poly-L-lysine (Sigma, St Louis, MO, USA). Transient expression of SOD1 plasmids (0.1 μg of DNA/well) and pcDNA3.1/His Max-Dorfin or pCMV-β (0.3 μg of DNA/well) in Neuro2a cells (2×10^4 cells/well) was accomplished with LipofectAMINE PLUS reagent (Invitrogen Corp.). After incubation for 3 h with transfection reagents, transfected cells were cultured in differentiation medium (DMEM supplemented with 1% fetal calf serum and 20 μM retinoic acid). To detect Xpress-Dorfin fusion protein, 0.5 μM proteasome inhibitor MG132 (Sigma) was added 16 h before collection, as described previously (Niwa *et al.* 2001).

Cell fractionation

At each time point (0, 24 and 48 h) after transfection, cells were collected and gently homogenized with a Dounce homogenizer in cold buffer [250 mM sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM EDTA and protease inhibitor cocktail (Complete Mini EDTA-free; Roche Diagnostics, Basel, Switzerland)]. Cell fractionation was performed as described previously (Takeuchi *et al.* 2002a). To verify the fractionation, each fraction was subjected to western blotting for cytochrome *c* oxidase (COX) as a mitochondrial marker using anti-COX subunit IV mouse monoclonal antibody (1 : 1000; Molecular Probes, Eugene, OR, USA), and β-actin as a cytosolic marker using anti-β-actin mouse monoclonal antibody (1 : 5000; Sigma).

Western blot analysis

The protein concentration was determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and western blotting was done as described previously (Takeuchi *et al.* 2002b). To evaluate the level of mitochondrially localized SOD1-EGFP fusion proteins, 20 μg protein from the mitochondrial fraction was loaded. For analyzing the release of cytochrome *c* from the mitochondria into the cytosol, 20 μg protein from the mitochondrial fraction or the cytosolic fraction was loaded.

To assess the levels of SOD1-EGFP fusion proteins, Xpress-Dorfin fusion proteins and the activation of caspase-9 and caspase-3, cells were collected at each time point (0, 24 and 48 h) after transfection, and lysed in TNES buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 0.1% sodium dodecyl sulfate and protease inhibitor cocktail) as described previously (Takeuchi *et al.* 2002a). For the analysis, 20 μg protein from the total lysate was loaded.

The primary antibodies used were as follows: anti-SOD1 rabbit polyclonal antibody (1 : 10 000; StressGen Biotechnologies, Victoria, BC, Canada), anti-Xpress mouse monoclonal antibody (1 : 5000; Invitrogen Corp.), anti-caspase-3 rabbit polyclonal antibody and anti-caspase-9 rabbit polyclonal antibody (1 : 1000; Cell Signaling, Beverly, MA, USA) and anti-cytochrome *c* mouse monoclonal antibody (1 : 1000; Pharmingen, San Diego, CA, USA). After overnight incubation with primary antibodies at 4°C, each blot was probed with horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG (1 : 5000; Amersham Biosciences, Piscataway, NJ, USA). Blots were then visualized with ECL Plus western blotting detection reagents (Amersham Biosciences). The signal intensity was quantified by densitometry using NIH Image 1.63 software.

Immunocytochemistry

At each time point (0, 24 and 48 h) after transfection, cells were fixed with 4% paraformaldehyde for 30 min on ice and then

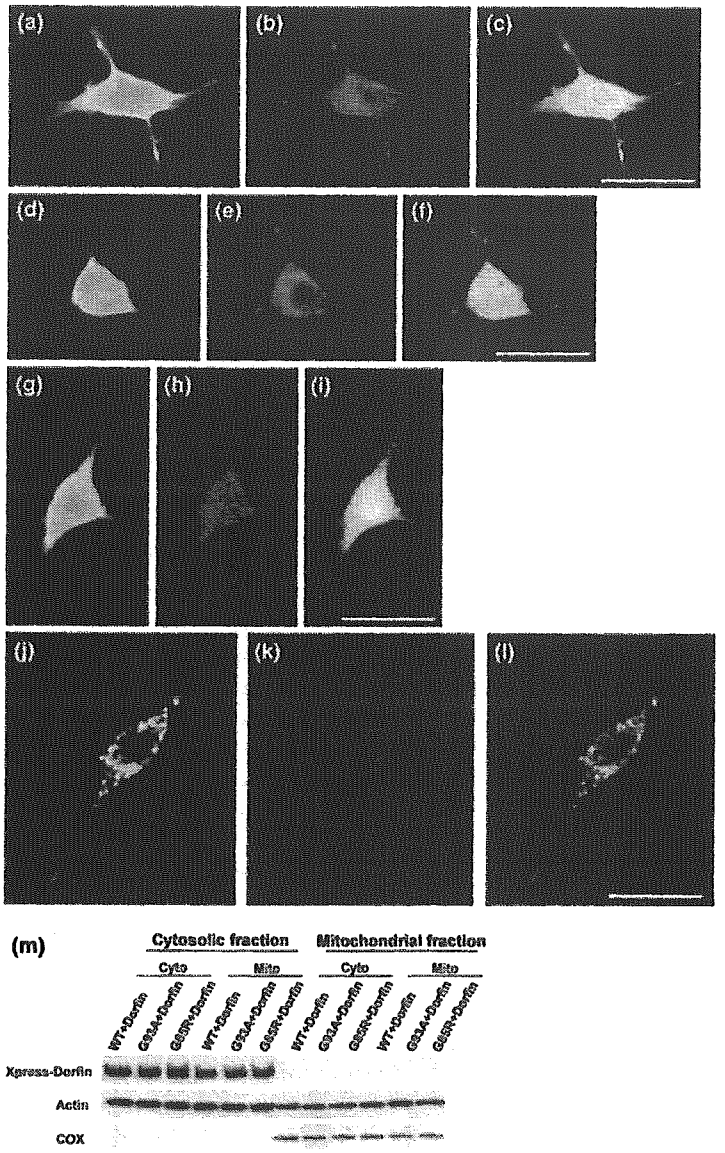

Fig. 1 Subcellular localization of SOD1-EGFP and Xpress-Dorfin in Neuro2a cells. (a–l) Confocal laser scanning microscopic images at 48 h after transfection. (m) Fractionation analysis of Xpress-Dorfin fusion protein. (a–c) Cyto-WT + Xpress-Dorfin, (d–f) Cyto-G93A + Xpress-Dorfin, (g–l) Cyto-G85R + Xpress-Dorfin; (j–l) Mito-G93A + Xpress-Dorfin. SOD1-EGFP fusion proteins (green; a, d and g) and Xpress-Dorfin fusion proteins (red; b, e and h) were observed ubiquitously in the cells with Cyto-SOD1 containing no organelle-oriented signals. SOD1-EGFP fusion proteins and Xpress-Dorfin fusion proteins were co-localized (yellow; c, f and i). In contrast, in the cells with Mito-SOD1, SOD1-EGFP fusion proteins were observed in the mitochondria (green; j) and Xpress-Dorfin fusion proteins (red; k) were observed mainly in the cytoplasm. They were not co-localized in the cells with Mito-SOD1 (l). Cells were counterstained with TO-PRO-3 (blue). Scale bars, 10 μm. Western blots also revealed that Xpress-Dorfin fusion proteins were absent in the mitochondrial fraction (m).

permeabilized with 0.05% Triton X-100 at room temperature for 10 min. They were stained with the anti-Xpress mouse monoclonal antibody (1 : 5000; Invitrogen Corp.) at 4°C overnight. They were subsequently stained with Alexa-568-conjugated secondary antibody (1 : 5000; Molecular Probes) at room temperature for 90 min. Then they were counterstained with 2 μg/mL TO-PRO-3 (Molecular Probes) at room temperature for 10 min, and mounted in Gelvatol. A confocal laser scanning microscope (MRC1024; Bio-Rad Laboratories) was used for the morphological analysis.

Quantitative assessment of mitochondrial impairment and cell death

To assess cell viability through mitochondrial impairment, we used the 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay with CellTiter 96 Aqueous one solution assay (Promega, Madison, WI, USA), as described previously (Takeuchi *et al.* 2002a). At each time point (0, 24 and 48 h) after transfection, MTS assays were carried out in six independent trials. Absorbance at 490 nm was measured in a multiple plate reader as described previously (Ishigaki *et al.* 2002).

Cell death was assessed by the dye exclusion method with propidium iodide (PI; Molecular Probes) as described previously (Takeuchi *et al.* 2002a). At each time point (0, 24 and 48 h) after transfection, cells were incubated with 2 μg/mL PI in DMEM for 15 min at room temperature and mounted in Gelvatol. More than 200 transfected cells in duplicate slides were assessed blindly in three independent trials under a conventional fluorescent microscope. The ratio of dead cells was calculated as a percentage of PI-positive cells among EGFP-positive cells.

Statistical analysis

All results were analyzed by two-way ANOVA with Tukey–Kramer post-hoc test, using Statview software version 5 (SAS Institute Inc., Cary, NC, USA).

Results

Dorfin reduces the levels of total, cytosolic and mitochondrial mutant SOD1

Confocal laser scanning microscopic images revealed that expression of both non-organelle-oriented Cyto-SOD1 plasmid and pcDNA3.1/HisMax-Dorfin was diffusely present in the cells. SOD1-EGFP fusion proteins were co-localized with Xpress-Dorfin fusion proteins (Figs 1a–i), consistent with our previous study (Niwa *et al.* 2002; Takeuchi *et al.* 2002a). In contrast, the expression of mitochondria-oriented Mito-SOD1 plasmid was observed in the mitochondria, as in our previous report (Takeuchi *et al.* 2002a), and was not co-localized with Xpress-Dorfin fusion proteins (Figs 1j–l). Western blots also revealed that Xpress-Dorfin fusion proteins were absent from the mitochondrial fraction (Fig. 1m). At 48 h after transfection, co-expression of Dorfin had reduced the total cell lysate level of SOD1-EGFP fusion proteins expressed by Cyto-G93A or Cyto-G85R by approximately 40%, whereas it did not affect those expressed by Cyto-WT (Fig. 2). In contrast, the amount of SOD1-EGFP fusion proteins expressed by Mito-SOD1 did not show any reduction even with co-expression of Dorfin (Fig. 2). In the cytosolic fraction, co-expression of Dorfin also reduced the level of SOD1-EGFP fusion proteins expressed by Cyto-G93A or Cyto-G85R by approximately 40%, whereas it did not affect those expressed by Cyto-WT (Fig. 3). As we described previously (Takeuchi *et al.* 2002a), cells with Mito-SOD1 showed very small amounts of SOD1-EGFP fusion proteins in the cytosolic fraction (Fig. 3). In the mitochondrial fraction, co-expression of Dorfin also reduced the level of SOD1-EGFP fusion proteins expressed by Cyto-G93A or Cyto-G85R by approximately 50%, whereas it did not affect those expressed by Cyto-WT (Fig. 4). This reduction in mitochondrial SOD1-EGFP was observed from 24 h after transfection, earlier than that of total or cytosolic SOD1-EGFP. In contrast, in the cells with Mito-SOD1, Dorfin did not reduce the amount of mitochondrial SOD1-EGFP fusion proteins (Fig. 4). The above results suggest that the mitochondrial accumulation of mutant SOD1 without organelle-oriented signals might be a result of mutant SOD1 in the cytosol, and we suggest that Dorfin, a cytosolic E3, reduced the accumulation of mutant SOD1 in the mitochondria by enhancing the degradation of mutant SOD1 in the cytosol, not in the mitochondria.

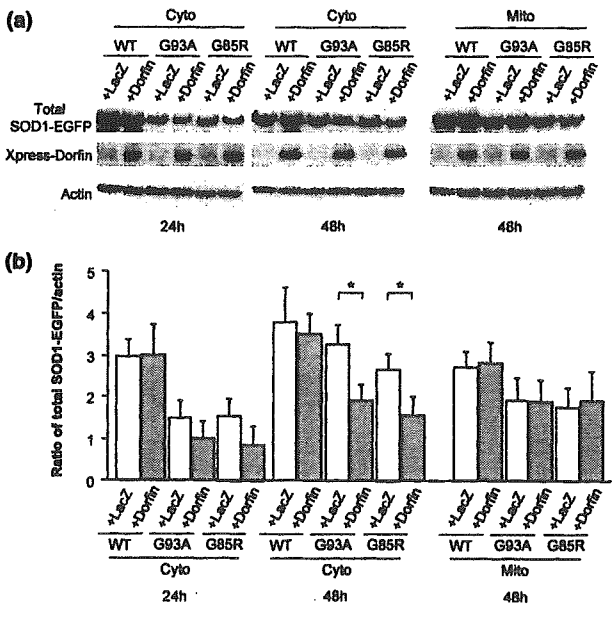

Fig. 2 Level of total SOD1-EGFP fusion protein. (a) Levels of total SOD1-EGFP fusion protein and Xpress-Dorfin fusion protein. (b) Densitometric analysis of total SOD1-EGFP fusion protein expressed as a ratio to actin. Dorfin significantly reduced the level of total SOD1-EGFP fusion protein expressed by Cyto-G93A or Cyto-G85R, whereas it did not reduce that expressed by Mito-SOD1. Values are mean ± SD ($n = 4$). *$p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

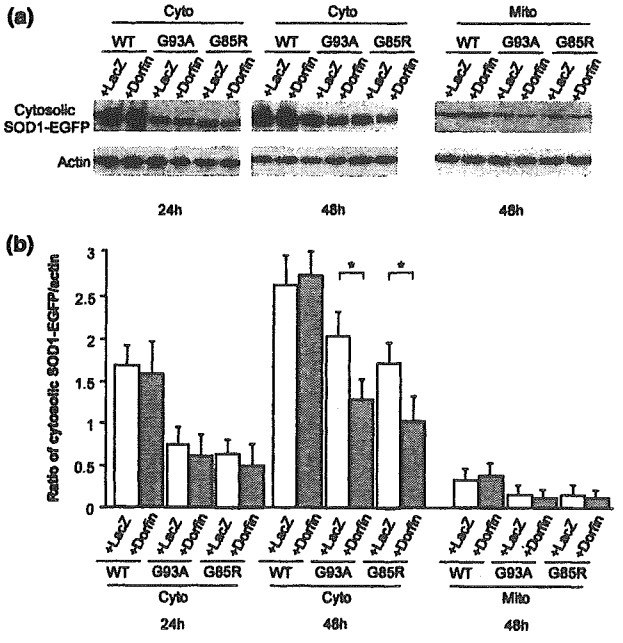

Fig. 3 Level of cytosolic SOD1-EGFP fusion protein. (a) Levels of cytosolic SOD1-EGFP fusion protein. (b) Densitometric analysis of cytosolic SOD1-EGFP fusion protein expressed as a ratio to actin. In the cytosolic fraction, Dorfin significantly reduced the levels of SOD1-EGFP fusion protein expressed by Cyto-G93A or Cyto-G85R. Mito-SOD1 showed very small amounts of SOD1-EGFP fusion proteins in the cytosolic fraction. Values are mean ± SD ($n = 4$). *$p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

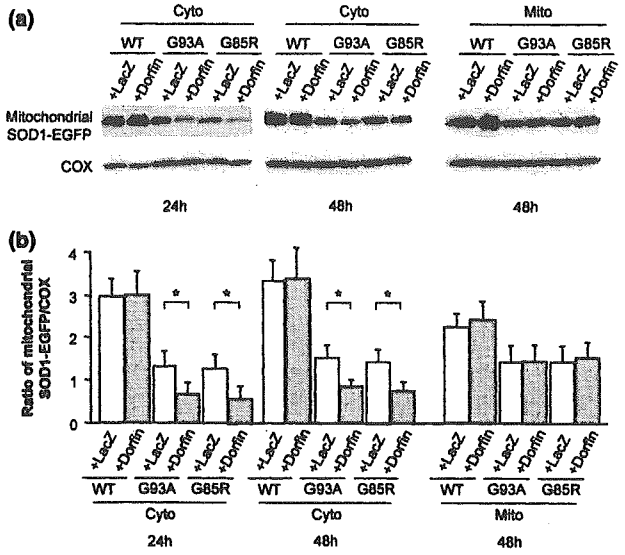

Fig. 4 Level of mitochondrial SOD1-EGFP fusion protein. (a) Levels of mitochondrial SOD1-EGFP fusion protein. (b) Densitometric analysis of mitochondrial SOD1-EGFP fusion protein expressed as a ratio to COX. In the mitochondrial fraction, Dorfin significantly reduced the level of SOD1-EGFP fusion protein expressed by Cyto-G93A or Cyto-G85R, whereas it did not reduce that expressed by Mito-SOD1. Values are mean ± SD ($n = 4$). $^*p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

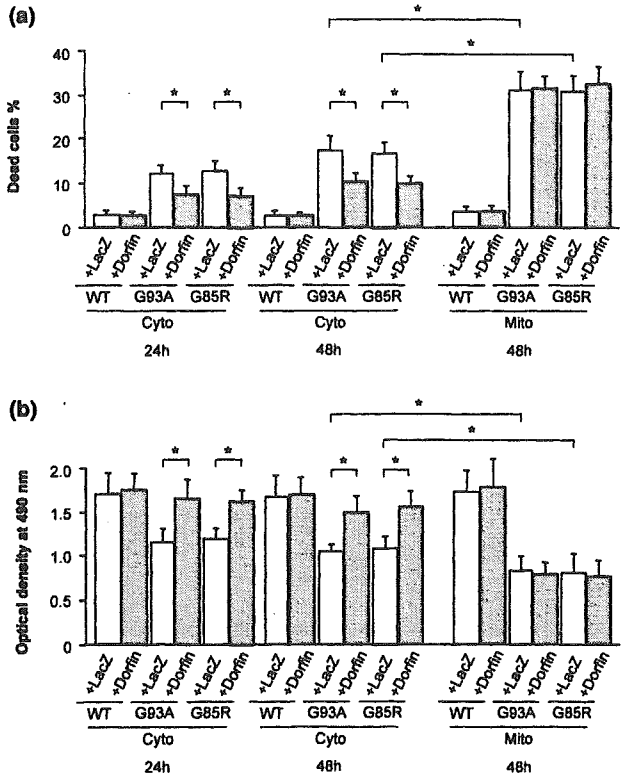

Fig. 5 (a) Frequency of dead cells and (b) mitochondrial impairment analyzed by MTS assay. The cells with Mito-G93A and Mito-G85R exhibited a significantly higher level of cell death and mitochondrial impairment than those with Cyto-G93A and Cyto-G85R. Dorfin significantly decreased cell death and mitochondrial impairment induced by Cyto-G93A and Cyto-G85R, whereas it did not affect those induced by Mito-SOD1. Values are mean ± SD ($n = 6$). $^*p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

Dorfin protects neuronal cells from mutant SOD1-mediated neurotoxicity by reducing mitochondrial mutant SOD1

As we demonstrated previously (Takeuchi *et al.* 2002a), the cells with Cyto-G93A and Cyto-G85R underwent cell death (Fig. 5a) and mitochondrial impairment (Fig. 5b), whereas those with Cyto-WT did not. The cells with Mito-G93A and Mito-G85R exhibited significantly more cell death and mitochondrial impairment than those with Cyto-G93A and Cyto-G85R, whereas those with Mito-WT did not (Fig. 5). Co-expression of Dorfin significantly ameliorated cell death and mitochondrial impairment induced by Cyto-G93A and Cyto-G85R (Fig. 5), as in our previous report (Niwa *et al.* 2002). In contrast, Dorfin did not affect cell death and mitochondrial impairment induced by Mito-SOD1 (Fig. 5), whose protein level Dorfin did not reduce. These findings suggest that Dorfin ameliorates mutant SOD1-mediated neurotoxicity by reducing the accumulation of mutant SOD1 in the mitochondria.

Dorfin reduces mitochondrial cytochrome *c* release and sequential activation of caspase-9 and caspase-3

We next assessed whether Dorfin reduced the mitochondrial death signal associated with the mutant SOD1-mediated cytotoxicity. Western blots revealed that Cyto-G93A and Cyto-G85R induced a gradual increase in the cytochrome *c* released from the mitochondria into the cytosol, whereas Cyto-WT did not (Fig. 6). The cells with Mito-G93A and Mito-G85R also exhibited a higher level of cytochrome *c* release than those with Cyto-G93A and Cyto-G85R, whereas those with Mito-WT did not (Fig. 6). Co-expression of Dorfin significantly reduced the release of cytochrome *c* from the mitochondria into the cytosol induced by Cyto-G93A and Cyto-G85R (Fig. 6). In the cells with Mito-G93A and Mito-G85R, however, Dorfin did not reduce the cytochrome *c* release from the mitochondria into the cytosol (Fig. 6).

Next, we examined whether Dorfin affected the downstream signal cascade of the activation of caspase-9 and caspase-3 following the release of mitochondrial cytochrome *c*. As we demonstrated previously (Takeuchi *et al.* 2002a), western blots revealed that Cyto-G93A and Cyto-G85R induced gradual activation of caspase-9 and caspase-3, whereas Cyto-WT did not (Figs 7 and 8). The cells with Mito-G93A and Mito-G85R exhibited a higher level of activation of caspase-9 and caspase-3 than those with Cyto-G93A and Cyto-G85R, whereas those with Mito-WT did not (Figs 7 and 8). Co-expression of Dorfin significantly reduced

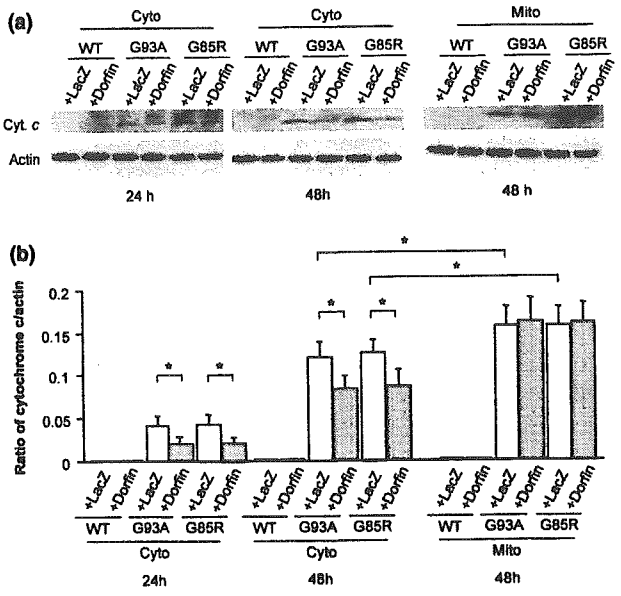

Fig. 6 Western blot analysis of cytochrome *c* release. (a) Time course of mitochondrial cytochrome *c* release into the cytosol. (b) Densitometric analysis of cytochrome *c* release expressed as a ratio to COX. The cells with Mito-G93A and Mito-G85R exhibited significantly more cytochrome *c* release than those with Cyto-G93A and Cyto-G85R. Dorfin significantly reduced the amount of mitochondrial cytochrome *c* released into the cytosol induced by Cyto-G93A and Cyto-G85R, whereas it did not affect that induced by Mito-SOD1. Values are mean ± SD ($n = 4$). $^*p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

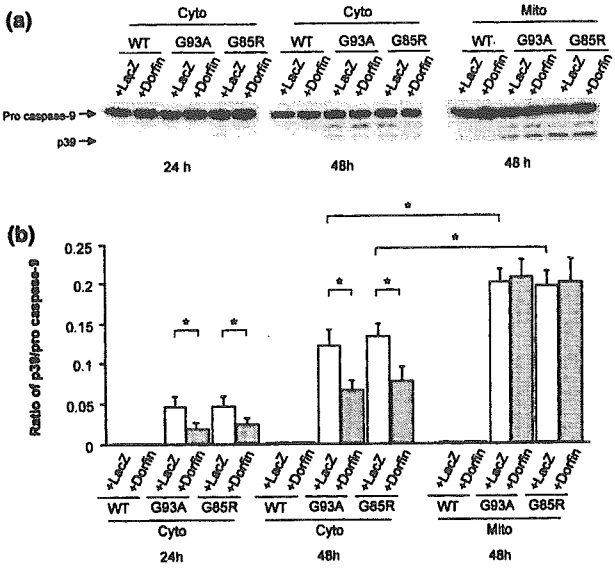

Fig. 7 Western blot analysis of caspase-9 activation. (a) Time course of the activation of caspase-9. (b) Densitometric analysis of caspase-9 activation. The cells with Mito-G93A and Mito-G85R exhibited significantly more activation of caspase-9 than those with Cyto-G93A and Cyto-G85R. Dorfin significantly reduced the activation of caspase-9 induced by Cyto-G93A and Cyto-G85R, whereas it did not reduce that induced by Mito-SOD1. Values are mean ± SD ($n = 4$). $^*p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

the activation of caspase-9 and caspase-3 induced by Cyto-G93A and Cyto-G85R (Figs 7 and 8). However, Dorfin did not reduce the activation of caspase-9 and caspase-3 induced by Mito-G93A and Mito-G85R (Figs 7 and 8), as it did not reduce the release of cytochrome *c* induced by Mito-G93A and Mito-G85R (Fig. 6). These findings combined with the aforementioned observations suggest that the reduction in the amount of mitochondrial mutant SOD1 due to Dorfin results in attenuated activation of the mitochondrial PCD pathway and prevents eventual cell death.

Discussion

In the present study, we first demonstrated that Dorfin, an E3 for mutant SOD1s, attenuated the activation of the mitochondrial PCD pathway and prevented eventual cell death in a neuronal cell model of FALS by reducing the amount of mutant SOD1 in the mitochondria. Dorfin reduced the levels of both cytosolic and mitochondrial mutant SOD1-EGFP fusion proteins that were expressed by Cyto-G93A and Cyto-G85R without organelle-oriented signals, whereas Dorfin did not affect the level of mutant SOD1-EGFP fusion protein that was expressed by Mito-G93A and Mito-G85R with mitochondrial localizing signals. The reduction in the level of

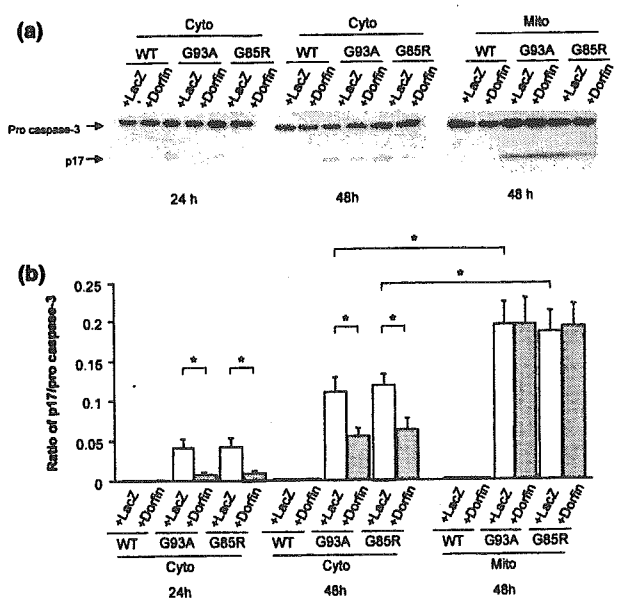

Fig. 8 Western blot analysis of caspase-3 activation. (a) Time course of activation of caspase-3. (b) Densitometric analysis of caspase-3 activation. The cells with Mito-G93A and Mito-G85R exhibited significantly more activation of caspase-3 than those with Cyto-G93A and Cyto-G85R. Dorfin significantly reduced the activation of caspase-3 induced by Cyto-G93A and Cyto-G85R, whereas it did not reduce that induced by Mito-SOD1. Values are mean ± SD ($n = 4$). $^*p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

mitochondrial SOD1-EGFP was observed earlier than that of total or cytosolic SOD1-EGFP. Moreover, Dorfin was present in the cytosol, not in the mitochondria. These findings indicated that the mitochondrial mutant SOD1 without organelle-oriented signals (Cyto-G93A and Cyto-G85R) might be translocated from the cytosol, and we suggest that Dorfin reduces the mitochondrial accumulation of mutant SOD1 by enhancing the degradation of mutant SOD1 in the cytosol through the ubiquitin–proteasomal pathway, thereby reducing the uptake of mutant SOD1 into the mitochondria.

Many reports have documented mitochondrial involvement in ALS and FALS. Mitochondrial degeneration with vacuolization or membrane disintegration in motor neurons is one of the earliest pathological findings in FALS Tg mice (Dal Canto and Gurney 1994; Wong *et al.* 1995; Hirano 1996; Kong and Xu 1998; Jaarsma *et al.* 2000; Higgins *et al.* 2003). Moreover, mitochondrial dysfunction such as altered calcium homeostasis (Carrì *et al.* 1997; Menzies *et al.* 2002b), decreased respiratory chain complex activity (Mattiazzi *et al.* 2002; Menzies *et al.* 2002a), alteration of mitochondria-related gene expression (Yoshihara *et al.* 2002) and an increase in reactive oxygen species (Beretta *et al.* 2003) have been reported in *in vitro* and *in vivo* models of FALS. Several studies have documented that SOD1, which has been considered a cytosolic enzyme, also exists in the mitochondrial intermembrane space (Okado-Matsumoto and Fridovich 2001; Sturtz *et al.* 2001; Higgins *et al.* 2002) and that the mitochondrial vacuoles are lined with mutant SOD1 in a FALS Tg mice model (Jaarsma *et al.* 2001; Higgins *et al.* 2003). Although the mitochondria-oriented vector we used here is designed to localize proteins to the mitochondrial matrix, we predict that SOD1-EGFP also exists in the mitochondrial intermembrane space through the process of its uptake into the mitochondrial matrix in our model, although were not able to confirm this. Recent studies also revealed that SOD1 in the mitochondria originates from the uptake of SOD1 in the cytosol (Sturtz *et al.* 2001; Okado-Matsumoto and Fridovich 2002; Field *et al.* 2003). At least our result provided enough evidence that Dorfin interacts with mutant SOD1 in the cytosol, not in the mitochondria. Thus we suggest that Dorfin indirectly reduces the mitochondrial accumulation of mutant SOD1 by reducing the uptake of mutant SOD1 into the mitochondria.

Previous studies demonstrated that the mitochondrial PCD pathway, cytochrome *c* release and subsequent caspase activation, might contribute to the motor neuron cell death in FALS (Durham *et al.* 1997; Martin 1999; Li *et al.* 2000; Pasinelli *et al.* 2000; Guégan *et al.* 2001; Kriz *et al.* 2002; Zhu *et al.* 2002). Thus, inhibiting the activation of the mitochondrial PCD pathway is potentially useful in the treatment of FALS. Methods for this include inhibition of cytochrome *c* release by minocycline (Zhu *et al.* 2002; Kriz *et al.* 2002), co-expression of bcl-2 (Lee *et al.* 2001) or X-chromosome-linked inhibitor of apoptosis protein (Ishigaki *et al.* 2002), and treatment with a broad caspase inhibitor zVAD-fmk (Pasinelli *et al.* 2000; Takeuchi *et al.* 2002a) or a caspase-9 specific inhibitor zLEHD-fmk (Takeuchi *et al.* 2002a). In this study, we demonstrated that Dorfin reduces the amount of mitochondrial mutant SOD1, attenuates the activation of the mitochondrial PCD pathway and prevents eventual neuronal cell death. It is therefore possible that reducing the amount of mutant SOD1 in the mitochondria may be adopted as a new therapeutic strategy for mutant SOD1-associated FALS.

Recent studies have suggested that some E3s, including Dorfin, act in a quality-control system to degrade cytosolic or transmembranous unfolded abnormal proteins (Moynihan *et al.* 1999; Fang *et al.* 2001; Meacham *et al.* 2001; Murata *et al.* 2001; Yoshida *et al.* 2002). The mitochondria also have a quality-control system that depends on mitochondria-specific molecular chaperones and ATPases associated with diverse cellular activities (AAA) proteases such as chaperonin 60 (Gottesman *et al.* 1997), mitochondrial heat-shock protein 70 (Savel'ev *et al.* 1998), and homologs of Lon, Yme1p, ClpP and ClpX (Wang *et al.* 1993; Suzuki *et al.* 1997; Langer 2000; Shah *et al.* 2000; Kang *et al.* 2002; Röttgers *et al.* 2003). A recent study documented that the accumulation of unfolded abnormal proteins in the mitochondria itself up-regulated the nuclear gene expression encoding mitochondrial-specific molecular chaperones (Zhao *et al.* 2002). Even though the mitochondria are able to dispose of abnormal proteins, they appear to have limited capacity to do this. They also seem to release death signals when abnormal proteins overflow their disposing capacity. Combination therapy such as Dorfin and mitochondria-specific molecular chaperones or AAA proteases thus seems more effective. Further investigations are needed to develop this therapeutic avenue.

There remains the problem of how the mutant SOD1 induces the mitochondrial PCD pathway. One of our previous studies revealed that bcl-2 family pro-apoptotic proteins, such as Bax, Bak, Bid, Bad and Bim, and other mitochondrial death signals such as apoptosis-inducing factor (AIF) and second mitochondria-derived activator of caspase (Smac) were not involved in the neuronal cell death in our model (Takeuchi *et al.* 2002a). Other studies have reported that translocation of Bax and cleavage of Bid were associated with neuronal cell death in the FALS Tg mouse model (Guégan *et al.* 2001; 2002), but there is a possibility that the surrounding environment of motor neurons such as astrocytes, microglia or dying neurons might have been affected in these models. Moreover, we have indicated that a non-apoptotic form of PCD might contribute to neuronal cell death through the mitochondrial PCD pathway in our model (Takeuchi *et al.* 2002a). Another report also mentioned that a non-apoptotic type of PCD acting through the mitochondrial PCD pathway might underlie mutant SOD1-related neurotoxicity (Guégan and Przedborski 2003). Further *in vivo*

investigations are needed to shed light on the mechanism of mutant SOD1-mediated neuronal cell death.

In this study we demonstrated that Dorfin, an E3 for mutant SOD1s, significantly reduced the level of mutant SOD1 in the mitochondria, attenuated the subsequent activation of the mitochondrial PCD pathway and prevented eventual neuronal cell death in a neuronal cell model of FALS. Reducing the accumulation of mutant SOD1 in the mitochondria may have an important place in the therapeutic strategy for mutant SOD1-associated FALS, and Dorfin may play a key role in this.

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Nerve excitability properties in Charcot–Marie–Tooth disease type 1A

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Summary

Charcot–Marie–Tooth disease type 1A (CMT1A) is commonly considered a prototype of a hereditary demyelinating polyneuropathy. Apart from the myelin involvement, there has been little information on axonal membrane properties in this condition. Taking advantage of the uniform nature of the disease process, we undertook the *in vivo* assessment of multiple axonal excitability properties at the median nerve in nine CMT1A patients with *PMP22* (peripheral myelin protein 22) gene duplication and 53 controls. The thresholds of CMT1A patients were much higher than normal, and threshold electrotonus (TE) exhibited a consistent pattern of abnormalities: early steep changes (fanning out) of both hyperpolarizing and depolarizing responses were followed by increased inward rectification to hyperpolarizing currents and unusually fast accommodation to depolarizing currents. Strength–duration time constants and the shapes of recovery cycles were normal, although refractoriness and superexcitability were reduced relative to controls. The high thresholds and early fanning out of electrotonus indicated altered cable properties, such that a greater proportion than normal of applied currents reached internodal rather than nodal axolemma. The rapid accommodation to depolarizing currents suggested activation of fast K^+ channels, which are normally sequestered from the nodal membrane. The excitability abnormalities are therefore consistent with a demyelinating pathology and exposure or spread of K^+ channels from under the myelin. It remains to be seen whether the TE abnormalities in CMT1A, which resemble previous recordings from normal immature rats, can be distinguished from those in acquired demyelinating neuropathies.

Keywords: Charcot–Marie–Tooth disease type 1A; paranode; membrane properties; threshold tracking; potassium channel

Abbreviations: CIDP = chronic inflammatory demyelinating polyneuropathy; CMAP = compound muscle action potential; CMT1A = Charcot–Marie–Tooth disease type 1A; CV = conduction velocity; DL = distal motor latency; PMP22 = peripheral myelin protein 22; SNAP = sensory nerve action potential; TE = threshold electrotonus

Introduction

Charcot–Marie–Tooth disease type 1A (CMT1A) is the most common form of hereditary motor and sensory neuropathy and its hallmark is diffuse demyelination (Dyck *et al.*, 1993; Birouk *et al.*, 1997). However, secondary axonal degeneration is common and its degree determines the patient's functional disability (Hattori *et al.*, 2003; Krajewski *et al.*, 2000; Hanemann and Gabreels-Festen, 2002). To date, the pathophysiology of the secondary axonal degeneration in CMT1 is unknown, although abnormal axon–Schwann cell interaction has been considered to play a major role (Sahenk and Mendell, 1999*a*; Kamholz *et al.*, 2000; Maier *et al.*, 2002). Intact Schwann cells are important in maintaining axonal integrity and development (Peles and Salzer, 2000; Martini, 2001; Scherer and Arroyo, 2002), so it would be reasonable to assume that in CMT1A abnormalities exist in axonal membrane properties, as well as in myelin.

Measurements of axonal excitability properties by threshold tracking have recently shed light on a variety of conditions affecting peripheral nerves (Bostock *et al.*, 1998; Burke *et al.*, 2001). The excitability properties are particularly sensitive to membrane potential, but also depend on nodal and internodal ion channels, as well as the passive

membrane properties, such a nodal width, and the extent to which the internodal axonal compartment is electrically isolated from the nodal compartment (Bostock *et al.*, 1998). Although many of these parameters are expected to be altered in demyelinating disease, several clinical studies have failed to reveal a clear-cut pattern of excitability changes related to demyelination. Thus a study of chronic inflammatory demyelinating polyneuropathy (CIDP) found raised thresholds but a shorter strength–duration time constant and no consistent changes in threshold electrotonus (Cappelen-Smith *et al.*, 2001). Studies of multifocal motor neuropathy have found evidence of membrane hyperpolarization distal to sites of conduction block (Kiernan *et al.*, 2002*b*), reduced Na^+ conductance (Priori *et al.*, 2002) and normal membrane properties proximal to sites of block (Cappelen-Smith *et al.*, 2002), but at the sites of conduction block, where demyelination has been reported (Kaji *et al.*, 1993), thresholds are very high and specific excitability changes relatable to demyelination have not been reported. A study of axonal and demyelinating forms of Guillain–Barré syndrome (Kuwabara *et al.*, 2002*a*) also failed to find any changes in nerve excitability properties at the wrist that could be directly related to the demyelination, probably because the major pathology occurred more distally in these patients. It has previously been argued that the reason why threshold electrotonus studies have failed to reveal consistent abnormalities in demyelinating neuropathies is because axons and nodes are affected non-uniformly, and fibres demyelinated at the point of stimulation will preferentially be excited at adjacent normal nodes, or other, more normal fibres will be excited in their place (Bostock *et al.*, 1998). This argument should be less applicable to CMT1A, in which it is possible to limit cases to a well-defined genetic defect [duplication of the *PMP22* (peripheral myelin protein 22) gene] and axons are affected relatively uniformly.

This study was therefore undertaken to test the hypothesis that CMT1A patients, unlike those with previously studied acquired demyelinating diseases, would exhibit a consistent pattern of abnormal excitability measures. A further aim was to test for secondary changes in axonal membrane properties, such as changes in membrane potential, which could not be related directly to altered myelination but which might be related to the secondary axonal degeneration. In the event, a consistent pattern of abnormal nerve excitability properties was found, which was consistent with demyelination, but there was little evidence of degeneration, or excitability changes that might be related to degeneration, in the sample of patients studied.

Patients and methods

Patients

Recordings were made from nine patients with genetically proven CMT1A (aged 11–75 years; mean 48.1 years; seven males and two females) from three university hospitals in Japan. All patients showed typical but variable clinical features of CMT type 1, such as diffuse areflexia/hyporeflexia, length-dependent sensory loss, distal atrophy and foot deformities. A fluorescence *in situ* hybridization-based assay identified the 1.5 Mb duplication on chromosome 17p11.2–12 containing the *PMP22* gene in all the subjects. No patient had a past history of diabetes, connective tissue disease, malignancy, electrolyte abnormality or use of neurotoxic drugs or steroids. All the patients had a clear family history of similar symptoms and signs of autosomal dominant inheritance. All the patients (and a parent for a minor) gave informed consent to participation in the study. This study was performed in accordance with the principles embodied in the Declaration of Helsinki and the protocol was approved by institutional review boards of all participating hospitals.

Conventional nerve conduction studies

Nerve conduction studies were performed with percutaneous stimulating and recording electrodes. The distal motor latency (DL), motor nerve conduction velocity (CV) and compound muscle action potentials (CMAP) were elicited with distal and proximal stimulation from the median (in the wrist with 7 cm stimulating–recording distance, and in the elbow), ulnar (in the wrist with 7 cm stimulating–recording distance, and in the forearm) and tibial (in the ankle with 8 cm stimulating–recording distance, and in the knee) nerves. Sensory nerve action potentials (SNAPs) were recorded antidromically from the median, ulnar and sural nerves using surface recording electrodes and stimulating–recording distances of 13, 11 and 14 cm respectively.

Nerve excitability measures

Studies were performed using a recently described protocol (Kiernan *et al.*, 2000) designed to measure multiple nerve excitability parameters rapidly.

CMAPs were recorded from thenar muscles using surface electrodes over the abductor pollicis brevis on the dominant hand side, with the active electrode at the motor point and the reference on the proximal phalanx. The EMG signal was amplified (gain 1000, bandwidth 1.6 Hz to 2 kHz) and digitized by a computer (486PC) with an A/D board (DT2812; Data Translation, Marlboro, MA, USA) using a sampling rate of 10 kHz. Stimulus waveforms generated by the computer were converted to current with a purpose-built isolated linear bipolar constant-current stimulator (maximum output ± 100 mA). The stimulus currents were applied via non-polarizable electrodes (Unique Medical, Tokyo, Japan), with the active electrode over the median nerve at the wrist and the reference electrode 10 cm proximal over muscle. Stimulation and recording were controlled by QTRAC software (©Institute of Neurology, London, with multiple excitability protocol TRONDXM).

Test current pulses of 0.2 or 1 ms were applied at 0.8 s intervals, and were combined with suprathreshold condition-

Table 1 *Results of the nerve conduction studies*

Nerve	DL (ms)	CMAP amplitude (mV)	Motor CV (m/s)	SNAP amplitude (μV)	Sensory CV (m/s)
Median	8.5 (6.9–10.2)	4.7 (1.8–8.9)	22.3 (16–39)	2.1 (0–6.8)	22.4 (18–28; n^* = 5)
Ulnar	7.4 (5.9–9.1)	3.1 (0.9–4.2)	22.2 (15–38)	0.5 (0–3.0)	23 (20–26; n = 2)
Tibial	10.3 (6.5–12.1)	1.2 (0–3.2)	18.7 (13–35)		
Sural				1.47 (0–12)	23 (16–29; n = 2)

Data are mean (range). There were nine patients (seven men, two women), with mean age 48.1 years (range 11–75 years). n^* is the number of patients in whom CV was obtainable (i.e. presence of SNAP). DL = distal latency; CMAP = compound muscle action potential; CV = conduction velocity; SNAP = sensory nerve action potential.

ing stimuli or subthreshold polarizing currents as required. The polarizing, conditioning and test current pulses were all delivered through the same electrodes. The amplitude of the CMAP was measured from baseline to negative peak. For all tracking studies, the target CMAP was set to 40% of maximum. Skin temperature was recorded using an adhesive probe over the nerve, adjacent to the stimulation electrode, to monitor temperature close to the site where axonal excitability was tested. The sequence of recordings followed that previously described (Kiernan *et al.*, 2000). Stimulus–response curves were recorded separately for test stimuli of durations 0.2 and 1 ms. The stimuli were increased in 6% steps, with two responses averaged for each step, until three averages were considered maximal. The ratio between the 0.2 and 1 ms stimuli required to evoke the same response was used to estimate the strength–duration time constant of axons of different threshold. A target response was then set at 40% of the maximum and the 1.0 ms test stimuli adjusted automatically by the computer to maintain this peak CMAP amplitude. Proportional tracking was used, whereby the change in stimulus amplitude from one trial to the next was made proportional to the 'error', or the difference between the last response and the target response (Bostock *et al.*, 1998). The slope of the stimulus–response curve was used to set the constant of proportionality and to optimize the tracking efficiency. Prolonged subthreshold currents were used to alter the potential difference across the internodal as well as the nodal axonal membrane. The changes in threshold associated with these electrotonic changes in membrane potential normally have a similar time course and are known as threshold electrotonus (TE) (Bostock *et al.*, 1998). Threshold tracking was used to record the changes in threshold induced by 100 ms polarizing currents, set to 40% (depolarizing) and –40% (hyperpolarizing) of the control threshold current. Three stimulus combinations were tested in turn: (i) test stimulus alone (to measure the control threshold current); (ii) test stimulus + depolarizing conditioning current; and (iii) test stimulus + hyperpolarizing conditioning current. Threshold was tested at 26 time points (maximum separation 10 ms) before, during and after the 100 ms conditioning currents. Each stimulus combination was repeated until three valid threshold estimates were recorded, as judged by the response being within 15% of the target response or alternate responses being either side of the target. We checked for the lack of CMAP response in all the raw traces after applying only conditioning stimulation.

The current–threshold relationship was tested with 1 ms pulses at the end of 200 ms polarizing currents, which were altered in 10% steps from +50% (depolarizing) to –100% (hyperpolarizing) of the control threshold. As with the conventional TE protocol, stimuli with conditioning currents were alternated with test stimuli alone, and each stimulus combination was repeated until three valid threshold estimates had been obtained.

The final part of the protocol recorded the recovery of excitability following a supramaximal conditioning stimulus. These changes were recorded at 18 conditioning ($1/n$) test intervals, decreasing from 200 to 2 ms in approximately geometrical progression. Three stimulus combinations were tested in turn: (i) unconditioned test stimulus (of 1 ms duration) tracking the control threshold; (ii) supramaximal conditioning stimulus (1 ms duration) alone; and (iii) conditioning + test stimuli. The response to (ii) was subtracted on-line from the response to (iii) before the test CMAP was measured, so that the conditioning maximal CMAP did not contaminate the measured response when the conditioning–test interval was short. Each stimulus combination was repeated until four valid threshold estimates had been obtained.

Control data

For threshold tracking studies, control data were obtained from 53 healthy individuals with mean age 43.1 years (range 23–84 years) at Chiba University Hospital. Given the fact that additional control data from an 8-year-old girl (not included for analysis at the parent's request) has shown a similar trend of the nerve excitability properties to the adult controls, data from an 11-year-old CMT1A patient was included for the study. All subjects (and a parent for a minor) gave informed consent.

Data analysis

Values for the excitability measures obtained in the present study were compared with normative data. The Mann–Whitney *U* test or repeated measures ANOVA (analysis of variance) was used for comparison using SPSS 11.0J (Tokyo, Japan). TEd (5 ms), TEd (10–20 ms) and TEd (90–100 ms) were the mean threshold reductions at or between the specific

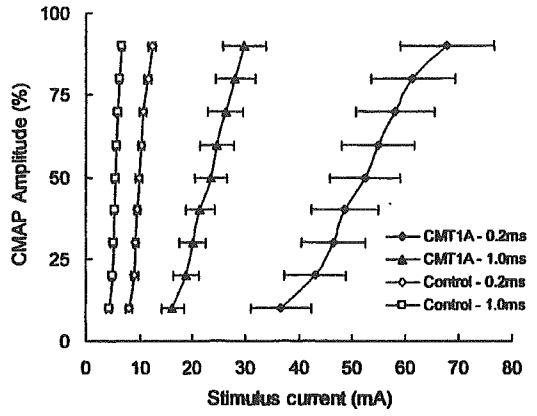

Fig. 1 Absolute stimulus–response curves (mean ± SEM) of median motor axons at the wrist in 53 healthy controls and nine CMT1A patients for two stimulus durations (0.2 and 1.0 ms). The threshold currents were significantly higher and the slopes of the curves were reduced, with greater variability of thresholds, in the patient group.

latencies after the onset of depolarizing current, and TEh (10–20 ms), TEh (20–40 ms) and TEh (90–100 ms) were the corresponding threshold changes after the onset of hyperpolarizing current. TEd (16 ms) – TEd (5 ms) was the difference in the threshold reductions at the respective latencies.

Results

Clinical features and nerve conduction study

Clinical profiles of the patients are shown in Table 1. Electrophysiological features showed diffuse demyelinative sensorimotor polyneuropathy with uniform conduction slowing, typical of CMT1, in all the subjects (Birouk *et al.*, 1997). Note that the median CMAP amplitudes were normal in 66% of the patients. As expected, there was an inverse relationship between age and median CMAP amplitude ($r = -0.61$, $y = -0.0637x + 7.9344$), but otherwise no age effect was observed in the analyses described below.

It has been shown that serum potassium level significantly affects axonal excitability (Kiernan *et al.*, 2002*a*; Kuwabara *et al.*, 2002*b*); the level was obtained in six of the nine subjects and all values were within normal limits (mean 4.1 mEq/l, range 3.7–5.3 mEq/l, normal, 3.5–5.5 mEq/l). As there was no significant change in the parameters assessed below between those from all the CMT patients and from patients with normal serum potassium levels, the remaining statistical analyses compared all the CMT patients with the controls.

Multiple excitability measures using threshold tracking

Stimulus–response curves

In the stimulus–response curves, the threshold currents in the nine CMT1A patients were significantly higher than those in the 53 healthy controls (Fig. 1). The stimulation current required to produce a minimal (10%) CMAP in the CMT1A patients was more than three times as high as that required to produce a maximal CMAP in healthy controls (Fig. 1). To produce a CMAP 40% of maximum, the mean absolute current for the 0.2 ms test stimulus was 48.7 ± 18.8 mA in the CMT1A patients and 9.5 ± 0.6 mA in the controls ($P < 0.001$). The mean absolute current for the 1.0 ms test stimulus was 21.5 ± 8.2 mA in the patients and 5.3 ± 0.5 mA in the controls ($P < 0.001$). Despite the significantly greater stimulation current in the patients, the test was well tolerated by the patients, possibly because of impaired sensation.

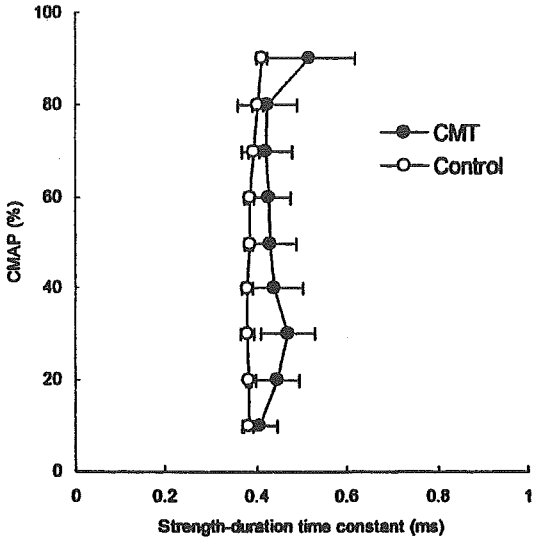

Fig. 2 The strength–duration time constant in CMT1A patients and controls (mean ± SEM). Although the strength–duration time constant was slightly longer in CMT1A than in controls, no significant change was demonstrated.

Strength–duration properties

Although the strength–duration time constant was slightly increased in the CMT group, there was no significant difference between the two groups (Fig. 2). The strength–duration time constant was fairly stable in both controls and CMT1A patients throughout the different CMAP amplitude levels. However, in CMT1A patients, an inverse relationship between the maximum CMAP amplitude and the strength–duration time constant at the 50% maximum CMAP level ($r = -0.51$) was found.

Recovery cycle

The patterns of the recovery cycles were similar in controls and CMT patients, the relative refractory period lasting <3 ms, supernormality being maximal at the 5-ms conditioning–test interval and late subnormality maximal at ~40 ms (Fig. 3). The extent of the changes in threshold during the refractory period was significantly greater in the control group at the 2-

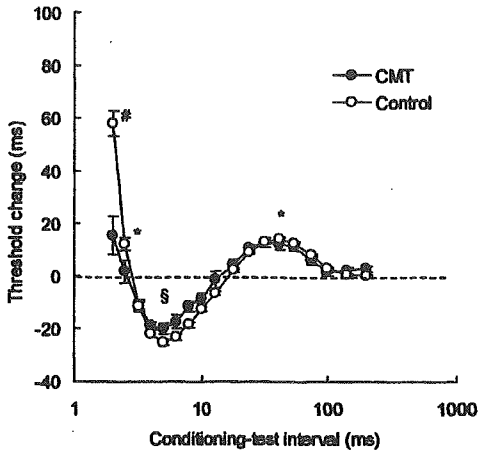

Fig. 3 The recovery cycle in CMT1A patients and controls (mean ± SEM). Nerves from patients with CMT1A showed less pronounced threshold changes in relative refractory and supernormal periods than controls, though durations of each period were similar. $P < 0.0007$; $^{\S}P < 0.02$; *not significant.

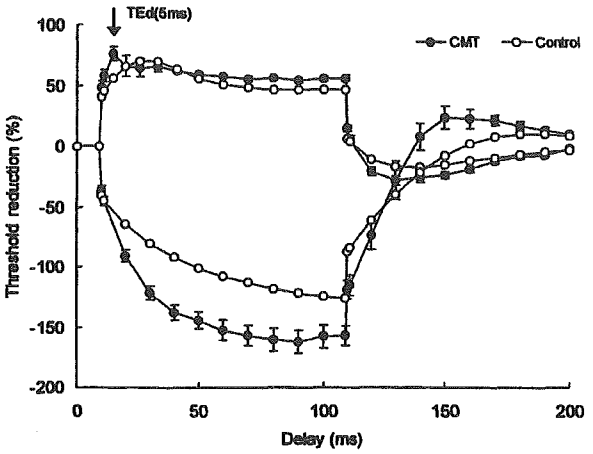

Fig. 4 Threshold electrotonus (mean ± SEM). In CMT1A patients, accommodation to depolarizing currents was stronger and occurred earlier than in controls. There was generalized widening out of the curves in CMT1A (fanning out) and greater accommodation to long hyperpolarizing currents.

ms conditioning–test interval ($P < 0.0007$), but not significantly different at the 2.5-ms interval ($P = 0.10$). For supernormality, the control group demonstrated a greater threshold change than the CMT group ($P < 0.02$), but there was no difference in late subnormality ($P = 0.88$) (Fig. 3). These findings of normal durations of the periods and reduced threshold changes in the patient group compared with those in controls are similar to the data in chronic inflammatory demyelinating polyneuropathy (Cappelen-Smith *et al.*, 2001).

Threshold electrotonus and current–voltage relationships

The most striking abnormalities in excitability parameters were revealed by the recordings of TE (Fig. 4). Table 2 documents comparisons of various excitability measures. Significant changes were observed in the responses to hyperpolarizing current. These were most pronounced in the early part of the responses [TEh (10–20 ms) and TEh (20–40 ms)] (Table 2) but still present at 90–100 ms. A closer look at the early part of the response to depolarizing current [TEd (10–20ms)] also disclosed steeper than normal changes. Because families of these electrotonus response curves can resemble the ribs of a Japanese fan, coming from a point near the origin of the plot (Fig. 6B), these changes can be described as a 'fanning-out' of the responses (Kaji, 1997). The more pronounced curvature in the CMT hyperpolarizing electrotonus suggests more accommodation due to activation of inward rectification by the hyperpolarization-activated current I_H (see Discussion).

The depolarizing electrotonus was more complicated, the CMT patients exhibiting first more, then less, and then more threshold reduction than the controls. At 5 ms, the depolarizing current induced a significantly greater threshold reduction than in controls (fanning-out). This was, however, quickly followed by an accommodative fall in threshold, between 5 and 16 ms. At longer delays, the CMT patients again showed a significantly greater threshold reduction than controls.

The current–threshold relationship (Fig. 5) also showed evidence of contrasting changes in passive and voltage-dependent membrane properties. With small currents, the slope of the current–threshold relationship was reduced, which, like the early fanning out of the TE, indicates that a greater fraction of the applied current was reaching the internodal axon (see Discussion). With larger currents, however, the slope was increased, in both the depolarizing and hyperpolarizing directions, until the absolute threshold changes returned towards and crossed the control curves respectively, indicating increased outward and inward rectification. Neither the TE nor the current–threshold relationship revealed correlation between CMAP amplitude and the extent of the nerve excitability abnormalities.

Discussion

The present study has shown that, in CMT1A, recognized as a polyneuropathy with uniform demyelination, there are consistent changes in nerve excitability properties, especially in resting thresholds and in TE. These changes were unlikely to be related to axonal degeneration, as CMAP amplitudes were fairly well preserved in the tested nerves, and there was no correlation between CMAP amplitude and the extent of the electrotonus abnormalities. Here we will consider the likely biophysical basis of the excitability changes observed.

Table 2 *Comparison of various excitability measures in TE*

	CMT1A (mean ± SD)	Control (mean ± SD)	*P*
TEd (10–20 ms)	65.5 ± 17.0	68.6 ± 4.9	0.73
TEd (90–100 ms)	56.1 ± 6.0	46.9 ± 4.8	< 0.001
TEh (10–20 ms)	−106.4 ± 15.2	−72.5 ± 6.5	< 0.001
TEh (20–40 ms)	−133.0 ± 16.3	−91.0 ± 10.6	< 0.001
TEh (90–100 ms)	−157.1 ± 25.7	−125.3 ± 24.1	< 0.004
TEd (5 ms)	76.2 ± 16.7	56.2 ± 3.7	< 0.001
TEd (16 ms) − TEd (5 ms)	−11.5 ± 23.9	14.0 ± 3.2	< 0.001

Values are threshold reduction (%).

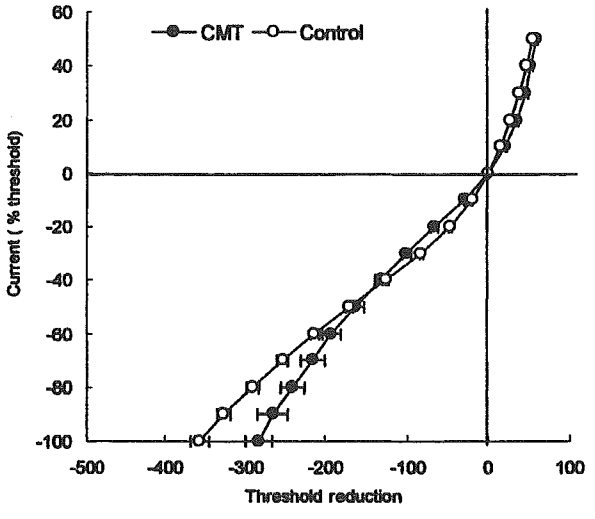

Fig. 5 The current–threshold relationship (mean ± SEM) demonstrated no statistical difference between the CMT1A and controls. However, the CMT group had a tendency to have greater threshold changes to depolarizing and weak hyperpolarizing currents consistent with fanning out in TE, and smaller changes in response to strong hyperpolarizing currents.

Increased resting threshold and early fanning out of threshold electrotonus

Figure 6 shows a simplified equivalent circuit for a segment of axon (node + internode), which accounts for the fast and slow phases of electrotonus and threshold (Barrett and Barrett, 1982; Yang *et al.*, 2000). It shows how an applied current (I) is divided into two components, a nodal component (I_n), which alters the potential on the nodal membrane by an amount I_nR_n (Ohm's law), and an internodal component (I_i), which initially changes the potential on the internodal axolemma at the rate I_i/C_i (as $C_i = I_i * t$). The initial proportion of current that crosses the nodal membrane (I_n/I) is set by the resistance ratio $R_{il}/(R_{il} + R_n)$, whereas the proportion crossing the internodal membrane (I_i/I) is $R_n/(R_{il} + R_n)$. The substantial increase in resting threshold current, and the increase in the early fanning out of TE indicate that in CMT1A the proportion of applied current crossing the nodal membrane is decreased and the proportion crossing the internodal membrane is correspondingly increased, i.e. that R_{il} is reduced in relation to R_n. If R_n were increased, the current threshold would fall, so we conclude that there is a reduction in R_{il}, which could be caused either by thin or 'leaky' myelin, or by a loosening of the axon–Schwann cell paranodal seal; both of these changes are consistent with the pathology of CMT1A. The reduction in R_{il} reduces I_n/I, so that the applied current has to be increased to reach the same threshold depolarization of the node. The reduction in R_{il} also increases I_i/I and the initial rate of polarization of the internodal axolemma. In a previous paper we introduced the idea of the 'fan' origin of TE (Bostock 1995; Kaji 1997; Yang *et al.*, 2000): the point (found by projecting back the tangents to the initial portion of slow electrotonus to the resting threshold) from which the slow electrotonus appears to originate (O in Fig. 6B). The time interval (t_f) from the fan origin to the time of current application was shown to be $C_iR_{il}(R_{il} + R_n)/R_n$ for the simplified circuit of Fig. 6A. A reduction in R_{il} reduces t_f and causes a more acute fanning-out of electrotonus.

Increased inward rectification in CMT1A

On the hyperpolarizing side, the early increased fanning out of TE in CMT1A is not maintained (Yang *et al.*, 2001). Inward rectification, most likely due to the slowly activating hyperpolarization-activated current I_H, sets in, so that by 100 ms the CMT1A and control curves are approaching each other, and by 200 ms (the time used for the current–threshold measurements in Fig. 5) the curves cross over for hyperpolarizing currents in excess of 40% of threshold. Greater activation of I_H during hyperpolarization TE in CMT1A patients is also indicated by the excitability overshoot after the end of the hyperpolarizing current at 150 ms, since I_H deactivates slowly. Although the TE and current–threshold data thus both indicate increased activation of I_H in CMT1A patients relative to normal controls, it is not clear whether this implies any alteration in axonal channel function (e.g. channel density). Because threshold currents are abnormally high in CMT1A, the polarizing currents used in the TE and current–threshold recordings are also abnormally high, so that the degree of hyperpolarization of the internodal axon must be much greater. It is therefore quite possible that the increased activation of I_H in CMT1A patients simply reflects

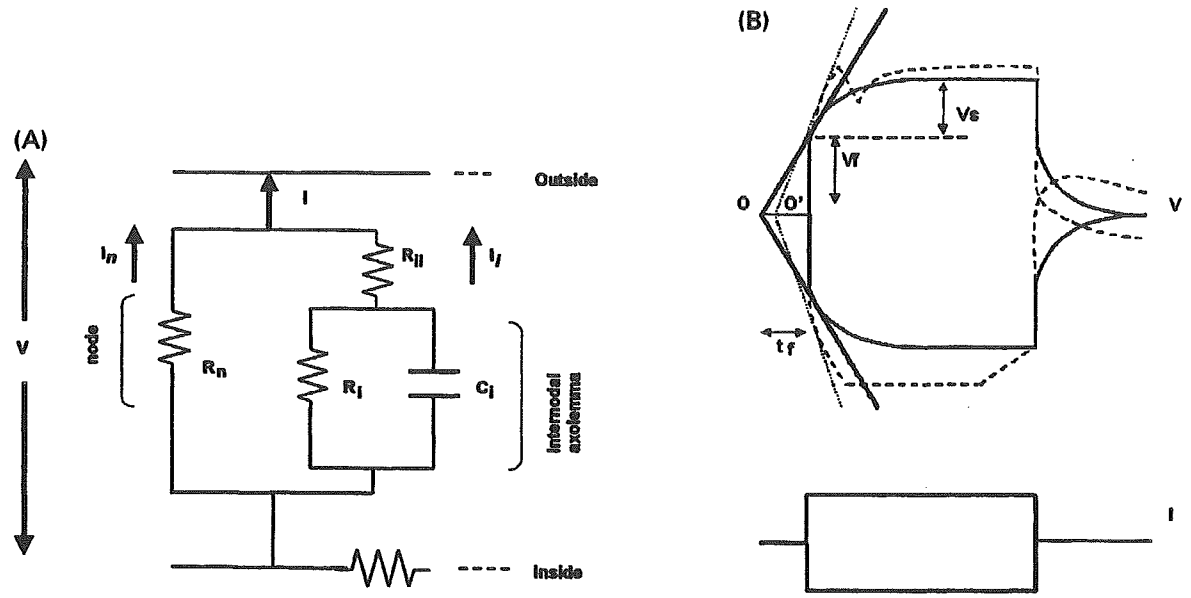

Fig. 6 A mathematical model of TE based solely on passive membrane properties. (A) A simplified equivalent circuit of myelinated axon (passive components only) (Barrett and Barrett, 1982), which generates fast and slow components of electrotonus. R_n, nodal resistance; R_{II}, internodal leakage resistance (access resistance to internodal axolemma, through and under the myelin sheath); R_i and C_i, resistance and capacitance of internodal axon. (B) Electrotonic changes in membrane potential (V) to long current pulses (I) predicted by the circuit in (A) in controls (solid lines) and CMT1A (dashed lines). V_f and V_s, fast and slow components of electrotonus; O and O′, apparent origins of fanning determined by lines tangential to the initial parts of the slow component; t_f, the time from O and O′ to the start of the current pulses. Note that the t_f is shorter in CMT1A than that in controls.

this increased membrane hyperpolarization rather than any abnormality in ion channels.

Rapid outward rectification in CMT1A

On the depolarizing side, the early increased fanning out of TE in CMT1A is much shorter-lived and quickly gives way to rapid accommodation. This rapid accommodation is similar to that previously seen in young rats, which was shown to be due to activation of fast K^+ channel channels that were blocked by 4-aminopyridine (Yang *et al.*, 2000). Whereas in normal, mature myelinated axons the fast K^+ channel channels $K_v1.1$ and $K_v1.2$ are concentrated in the juxtaparanodal region of the internode (Vabnick *et al.*, 1999; Girault and Peles, 2002), where they only affect slow components of electrotonus, in CMT1A they are activated rapidly by applied currents, either because they have spread to the nodal region, or because disruption of the axon–Schwann cell paranodal seal allows more current to reach the juxtaparanodal zone. Prior observations in the experimental demyelination demonstrated similar participation of the internodal K^+ channel channels to action potentials, in accordance with the present findings (Bostock *et al.*, 1981; Brismar, 1981; Chiu and Ritchie, 1981). An alternative viewpoint, suggested by the resemblance of TE in CMT1A patients to that in immature rats (Yang *et al.*, 2000), is that overproduction of the myelin protein PMP22 in the disease may inhibit normal nodal maturation. This interpretation is in accordance with studies demonstrating that axonal cytoskeleton in CMT1A is similar to those in immature axons (Sahenk *et al.*, 1999*b*), and that PMP22 overexpression in mice causes dysmyelination (Robaglia-Schlupp *et al.*, 2002).

Is membrane potential altered in CMT1A?

One of the aims of this study was to test for changes in axonal membrane properties which might be related to secondary axonal degeneration, such as membrane depolarization. Our recordings provide no evidence that membrane potential is appreciably abnormal in CMT1A. It might be argued that the raised thresholds and fanning out of TE are characteristics of membrane hyperpolarization. However, the early fanning out in CMT1A patients is very different from the fanning out seen in hyperpolarization, whether caused by DC currents, release of ischaemia, hypokalaemia or occurring in multifocal motor neuropathy (Kiernan and Bostock, 2000; Kiernan *et al.*, 2002*b*; Kuwabara *et al.*, 2002*b*), in which the deviation from normal increases during 100 ms hyperpolarization. In all of these examples of membrane hyperpolarization, superexcitability was increased relative to normal and relative to the late subexcitability. In CMT1A, however, the recovery cycle is relatively normal (Fig. 3), although refractoriness and superexcitability are significantly reduced. These changes in the recovery cycle are difficult to interpret in the presence of the substantial changes in passive membrane properties, but

are no more consistent with depolarization (which invariably increases refractoriness) than with hyperpolarization.

Comparison with other chronic demyelinating neuropathies

The results of the axonal excitability measures in the present study are different from those previously described in acquired demyelinating neuropathies, namely CIDP, multifocal motor neuropathy and Guillain–Barré syndrome, as described in the Introduction. Interestingly, a more recent study has found that a subset of CIDP patients, corresponding to those with a diffuse pattern of demyelination, exhibit features rather similar to those in CMT1A, namely increased thresholds, early fanning-out of TE, with increased activity of inward rectification on hyperpolarization (Sung *et al.*, 2004). There may be a contribution from endoneurial inflammation to the findings in these demyelinating neuropathies (Redford *et al.*, 1997), but it is unlikely that this plays a major role in CMT1A. It remains to be determined whether there are any consistent differences between the abnormal excitability properties in CMT1A and CIDP that could relate to the different aetiologies of demyelination.

Decreased nerve conduction velocity in CMT1A

Many factors may contribute to the decreased nerve conduction velocities typically seen in CMT1A, including axon diameter, myelin thickness and internodal distance. Recently, a few molecules, such as contactin, have been shown to play an important role in segregating nodes and juxtaparanodes and in anchoring Schwann cells to paranodes (Boyle *et al.*, 2001). The disruption of the paranodal junction alone in contactin mutant mice may account for the impaired conduction velocity (Boyle *et al.*, 2001). The disruption of the paranode is expected to reduce R_{il} (Fig. 6A), with consequent effects on excitability parameters, as found in CMT1A, as well as on conduction velocities (Boyle *et al.*, 2001; Kaji, 2003). An alternative explanation for the reduction in velocity is decreased Na^+ channel function (e.g. decreased density) (Kazarinova-Noyes *et al.*, 2001). However, our results did not support abnormal Na^+ channel function, as strength–duration time constant, a sensitive measure of persistent Na^+ current at the node, showed no significant change.

In summary, our data indicate that CMT1A patients exhibit a consistent pattern of abnormal nerve excitability properties. This pattern indicates increased access of applied currents to the internodal compartment of the axon and increased activation of fast K^+ channels. This is the first time that abnormal excitability properties have been found in a neuropathy that are logically attributable to altered myelination, and which may therefore aid the interpretation of excitability abnormalities in other conditions. However, the resemblance of the TE recordings to those in immature rats raises the possibility that the changes are related more specifically to nodal dysmaturity, and may differ in some respects from those in other demyelinating neuropathies.

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